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CYTOCHROMES OF DEVELOPING PLASTIDS OF GREENING BARLEY: EFFECTS OF INHIBITORS OF HAEM SYNTHESIS

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Key Word Index—*Hordeum vulgare*; Gramineae; etiolated barley; plastid cytochromes; laevulinic acid inhibition.

Abstract—The properties of the cytochromes of etiolated barley plastids and 20 hr greened barley plastids were examined by potentiometric titration. In etiolated plastics cytochrome f ($E_{m\,7.0} = 350$ mV), b_{LP} ($E_{m\,7.0} = 10$ mV) and b 563 ($E_{m\,7.0} = -20$ mV) were found. On greening the same three components were found, with almost the same mid-point potentials; in addition, cytochrome b_{HP} ($E_{m\,7.0} = 360$ mV) was found. Added laevulinic acid did not change the mid-point potentials of these cytochromes although it inhibited the development of cytochrome $b\,559_{HP}$ and cytochrome b 563, suggesting that newly synthesized haem was necessary to maintain cytochrome levels on greening. The content of cytochrome $b\,559_{LP}$ diminished and that of cytochrome f increased on greening.

INTRODUCTION

When seeds of higher plants germinate in the dark the shoots form no chlorophyll and contain etioplasts in place of the chloroplasts found in green tissue. Etioplasts contain nearly all the enzymes for CO_2 fixation [1], cytochrome f and some b-type cytochromes [2,3]. The nature of the b cytochromes found in fully greened chloroplasts is subject to dispute; most recently it has been shown [4] that lettuce chloroplasts contain three b-type cytochromes. These are $b\,559_{HP}$ ($E_{m\,7.0} = +370$ mV), $b\,559_{LP}$ ($E_{m\,7.0} = +20$ mV) and b 563 ($E_{m\,7.0} = -110$ mV). Cytochrome b 563 has been variously reported as having $E_{m\,7.0}$ ranging from 0 to -180 mV, with an n value of 1 or 2 [5–8]. We have examined the thermodynamic properties of the etioplast cytochromes of barley and made measurements to determine whether these change during development. This is a necessary study since it is known that $E_{m\,7.0}$ of at least one cytochrome, a cytochrome c, may vary when it is attached to an appropriate site on a membrane [9,10].

We have also examined the development of plastid cytochromes under conditions of haem limitation using the inhibitor laevulinic acid (LA), which is a competitive inhibitor of 5-aminolaevulinate (ALA) dehydratase [11].

RESULTS AND DISCUSSION

We have measured the E_m of plastid cytochromes in tissue from dark-grown, 20 hr-greened and laevulinic acid (LA)-treated barley. The results of one such series of oxidative–reductive titrations, for plastids prepared from the 20 hr-greened plants, are shown in Fig. 1, and the results for the whole series of determinations are shown in Table 1. Greening does not cause a change in the E_m of cytochrome f, b-563 or cytochrome b-559_{LP}, nor does inhibition of normal membrane development by treatment of greening shoots with LA. Cytochrome b-559_{HP} was present at concentrations too low to detect during redox titration of etiolated tissue; its concentration increased substantially after 20 hr greening. The changes in cytochrome content during greening are shown in Fig. 2. It can be seen that the major change is the rise in concentration of cytochrome b-559_{HP} from levels near 0 to around 0.28 nmol/mg protein at 24 hr.

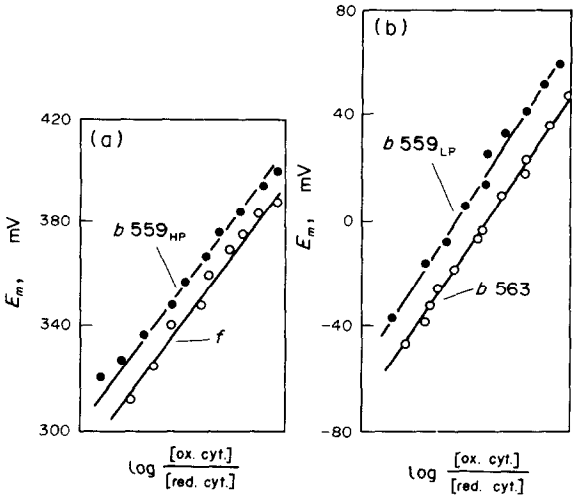

Fig. 1. Potentiometric titrations of the cytochromes of plastids from 20 hr-greened barley plants. Conditions as described in the Experimental. (a) Titration for cytochrome $b\,559_{HP}$ and cytochrome f; (b) titration for cytochrome $b\,559_{LP}$ and cytochrome b 563. The lines drawn are the theoretical lines for a one-electron redox reaction.

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Table 1. Mid-point potentials of cytochromes from dark-grown and 20 hr-greened, control and laevulinic acid-treated plants

	$E_{m7.0}$ (mV)			
	Etiolated		20 hr greened	
Cytochrome	Control	+LA	Control	+LA
f	350 ± 10	350 ± 10	356 ± 10	350 ± 15
b 559_{LP}	10 ± 10	10 ± 10	0 ± 20	0 ± 20
b 559_{HP}	—	—	360 ± 10	350 ± 10
b 563	−20 ± 10	−10 ± 10	−20 ± 10	−20 ± 10

Cytochrome *f* also rose during this period and its concentration almost doubled over 24 hr. Cytochrome *b*-559_{LP} showed a steady decline after the first 6 hr of illumination, in agreement with the findings of Plesnicar and Bendall [3] who determined the cytochrome content of fresh whole tissue. In contrast, it has been reported [2, 12] that in bean seedlings the concentration of all the plastid cytochromes increased on illumination of etiolated plants, although here cytochrome content was determined as molecules per plastid.

Inhibition of haem synthesis by the addition of LA affected the relative concentrations of cytochromes present in greening plastids (Table 2). The concentration of cytochrome *b*-559_{HP} was reduced by 50% and cytochrome *b*-563 by 33% and both cytochrome *f* and *b*-559_{LP} were slightly decreased. Spectroscopy at 77 K confirmed the decrease in cytochromes *b*-559_{HP} and cytochrome *b*-563 following LA treatment. It appears that LA is a useful inhibitor of the development of haem proteins as well as chlorophyll [13] and its action suggests that even during the greening period there is some turnover of cytochrome *b*-563 and *b*-559_{LP} as well as net synthesis of cytochrome *b*-559_{HP} and cytochrome *f*.

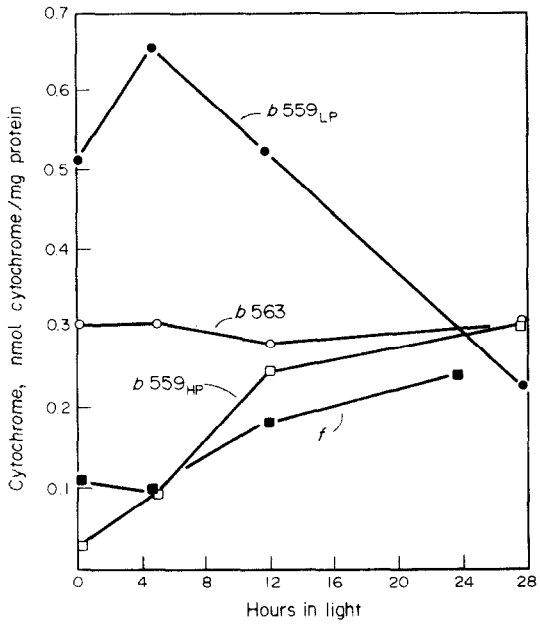

Fig. 2. Changes in cytochrome levels in isolated plastids prepared from etiolated barley illuminated intact for varying times. At the indicated time periods, plastids were isolated and cytochrome measurements made.

Previous work [13] has shown that the induction of nitrate reductase, which has a cytochrome *b*-like subunit [14], is severely inhibited by LA, to about 15% of normal values, although photosystem I and II activities were only slightly affected. In agreement with that result, we find that although the content of cytochromes is somewhat diminished by LA their thermodynamic and, presumably, kinetic properties are unaltered.

We find that $E_{m7.0}$ of all the cytochromes is the same in etioplasts as in chloroplasts despite the enormous changes in composition of the milieu of these membrane-bound components. The cytochrome mid-point potentials which we have determined in barley plastids are in reasonable agreement with those found by Rich and Bendall [4] for lettuce chloroplasts with the exception of cytochrome *b*-563 which was found [4] to have $E_{m7.0}$ of −110 mV in contrast to our value of −20 mV. Our value fits more closely with values reported elsewhere [7, 8]. It is possible that this large discrepancy arises because cytochrome *b*-563 is modified during plastid preparation or that it indeed has different thermodynamic properties in etioplasts and fully greened etioplasts. In such a complex mixture of cytochromes, absorption changes by other haem pigments cannot be excluded as a source of interference, although it is remarkable that these appear to manifest themselves only in the case of cytochrome *b*-563.

Table 2. Effect of laevulinic acid on the cytochrome content of plastids of greening barley

	Cytochrome content of plastids (nmol/mg protein)			
Sample	*f*	*b* 559_{LP}	*b* 559_{HP}	*b* 563
Control	0.2	0.31	0.06	0.18
+LA (40 mM)	0.17	0.26	0.03	0.12

Cut shoots were treated with 40 mM LA for 10 hr in light, following the 4 hr dark preincubation period. Plastids were prepared and cytochrome assayed as described in reference [16].

EXPERIMENTAL

Plant materials and application of inhibitors. Dark-grown barley (*Hordeum vulgare* L. var. Proctor) seedlings, 7–8 days old, were either illuminated intact or cut under H_2O and placed in beakers containing 10 mM K-Pi, pH 7.0. A pre-incubation period of 4 hr was allowed before the cut shoots were illuminated. Inhibitors were added to the buffer during this pre-incubation whenever necessary. A fan was placed in front of the beakers to facilitate uptake of the solutes.

Plastid preparation. Plastids were isolated from the barley shoots using the method of Griffiths [15].

Cytochrome measurements. Cytochrome concns in plastids were measured by difference spectroscopy as described in ref. [16].

Potentiometric titration. Titrations were performed in a dual wavelength spectrophotometer at room temp. using a stirred cuvette fitted with a Pt electrode, and gassed with O_2–free argon [17, 18]. The buffer used for the titrations contained 100 mM KCl and 20 mM MOPS, pH 7.0. The reference wavelength was 575 nm, sample wavelength was reduced and band maximum (554 nm for cytochrome *f*). Additions of dil. solns of dithionite and 100 mM ferricyanide were used in the reductive and oxidative titrations respectively. The mediators used were 25 μM hydroquinone ($E_{m\,7.0} = 280$ mV), 50 mM diaminodurol ($E_{m\,7.0} = 220$ mV), 25 μM phenazine methosulphate ($E_{m\,7.0} = 80$ mV), 25 μM phenazine ethosulphate ($E_{m\,7.0} = 55$ mV); 6 μM pyocyanine ($E_{m\,7.0} = -35$ mV) and 20 μM 2-hydroxy-1,4-naphthoquinone ($E_{m\,7.0} = -145$ mV). Data from the potentiometric titration at the appropriate wavelength maximum were replotted as described previously [18]. Oxidative and reductive titration points fitted the same curve.

Chlorophyll estimation. Chlorophyll was extracted from shoots and determined by the method of ref. [19].

Protein. Protein concns were determined using the method of ref. [20].

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